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Research Article

IN VITRO ANTIOXIDANT AND ANTIVIRAL ACTIVITY OF CAMEL MILK CASEIN HYDROLYSATES

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ABSTRACT. Camel milk casein and its hydrolysates obtained by pepsin enzyme action for 5 to 180 min, were assessed for antioxidant activities using 2, 2- diphenyl picryl-1- hydrazyl (DPPH), ferric reducing power, total antioxidant capacity (TAC) and β-carotene/linoleic acid bleaching tests. The antiviral effect against Coxsackie virus (Cox B6) was conducted *in vitro* by using A 549 and Vero cell lines. Coxsackie virus (Cox B6) was first interacted with casein and its hydrolysates at different concentrations, and then mixed with the different cell types. The viable cells were revealed by colorimetric method using neutral red. Results obtained suggest that pepsin camel milk casein hydrolysates can be exploited as a source of antioxidant compounds, and inhibit Coxsackie infection in both cell lines.

Keywords: *Camel milk casein, pepsin hydrolysates, antioxidant, Coxsackie virus, Vero, A549*

INTRODUCTION

Dromedary camel milk proteins composition is 3 to 3.9%, it contains two main groups: whey and casein, the second one constitutes 52 to 87% of total milk proteins, with main fractions: β casein (65%) followed by αS1 casein (21%) [1].

Within the protein sequence, peptides are inactive and require enzymatic proteolysis. Once released from the proteins precursor they become bioactive, and will have a positive impact on body, the beneficial health effects may be classified as antioxidative, antimicrobial, antithrombotic, antihypertensive or immunomodulatory [2, 3].

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS), with free radicals including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), they are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-

rays, ozone, cigarette smoking, air pollutants, and industrial chemicals [4]. They also react with proteins, hormones, enzyme molecules and even DNA, leading to damage cell membranes and the development of cancers. Antioxidants prevent free radical formation by scavenging them or by promoting their decomposition. In addition to natural antioxidants based on polyphenols, peptides that can utilize free radicals are identified in various hydrolysates of milk proteins [**5**], in order to replace synthetic antioxidants (butylated hydroxytoluene BHT, butylated hydroxyanisole BHA, propyl gallate…) used in food industry for extending the storage period of end products, but recently reported to be dangerous and imposing human health risks.

Among risks, tumor in human, whose many reports ascribed the link of viral infections with cancer [6]. Coxsackie viruses are RNA viruses belonging to the family picornaviridae and the genus enterovirus, divided into two groups A and B, at least 6 serotypes of group B are recognized (Coxsackie virus B) tend to infect heart, pleura, pancreas and liver causing pleurodynia, myocarditis and hepatitis; they are also causing non- specific febrile illnesses and upper respiratory tract disease [7]. Enteroviruses grow at a wide pH range (3- 10). After initial replication in the oropharynx, enteroviruses survive the acidic environment of the stomach [8, 9].

The aim of this work was to provide more information on the peptic camel milk casein hydrolysates antioxidant activity and to explore antiviral activity against Cox B6 on both African green monkey kidney cells (Vero cells) and adenocarcinomic human alveolar basal epithelial cells (A549 cells).

MATERIALS AND METHODS

Vero and A549 cells, Dulbecco's modified Eagle medium (DMEM) and Cox B6 were obtained from the National Referenced Laboratory, Pasteur Institute, Algiers, Algeria.

Fetal calf serum (FCS), DPPH, pepsin, ferric chloride, potasium ferricyanide, SDS, coomassie blue, TEMED, tween 40 were purchased from Sigma-Aldrich and ferrous sulfate, ammonium persulfate, sulfuric acid, sodium phosphate, ammonium molybdate, ammonium per sulphate and methanol were of analytical grade.

Sampling

Samples were collected in the morning from healthy female camels in a local farm at Laghouat region (Algeria) in bottles, and kept on ice during transportation to the laboratory and then stored at 4°C.

Casein preparation

Casein samples were prepared as follows: camel milk was defatted by centrifugation (3500×g, 20 min) at 4°C, then filtered. The filtrate was diluted with distilled water and pH was adjusted to 4.3 by the addition of 1M HCl, then centrifuged (5000×g, 30 min, 20 °C). The precipitated casein was separated from whey and washed with distilled water to remove any residue, then neutralized by 1M NaOH, precipitated again at pH 4.3 and washed, afterwards spread on filter paper, dried after removing water and lyophilized.

To estimate the concentration of casein, the Lowry protein assay was performed.

Casein hydrolysis

Camel milk casein was treated with pepsin according to the method of Parrot et *al.* [10]. A casein solution was prepared (20 mg/mL), pH adjusted to 2.0 with 1M HCl, then a 1g/L pepsin solution in 0.01M HCl was added to casein at enzyme/substrate ratio 1:200 (w: w). Camel casein was hydrolyzed by pepsin for 15, 30, 45, 60, 90, 120,150 and 180min at 37°C in water bath. The enzymatic hydrolysis was stopped by heating the reaction volume at 85°C for 5 min.

SDS PAGE analysis

SDS PAGE was performed on polyacrylamide gel system described by LaemmLi **[11]**: 4.8% w/v polyacrylamide stacking gel at pH 6.8 and 17% polyacrylamide separation gel at pH 8.8. Samples were diluted in sample buffer containing stacking gel buffer, 10% SDS, β-mercaptoethanol then were heated at 95°C for 5 min.

10 µl of samples with 2 mg/mL protein was loaded onto the gel for electrophoresis at 200 V at 25mA constant current. After running the electrophoresis unit, proteins are fixed with 12% (w/v) trichloroacetic acid (TCA) for 30 min and then stained with 0.2 g of R-250 Coomassie brilliant blue dissolved 45 min in a mixture of methanol/acetic acid/water (90:20:90, v/v/v), followed by distaining in methanol/acetic acid/glycerol/water (90:10:10:90, v/v/v/v).

Antioxidant activity evaluation

Reducing power assay

The reducing power was determined according to the method described by Hwang et al. [12]. The hydrolysate aliquot (0.5 mL) was mixed with 0.5 mL 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1.0 % potassium ferricyanide ($K_3Fe(CN)_6$) and then incubated in a water bath at 50°C for 20 min. After cooling down, 0.5 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, then the mixture was centrifuged at 1500 g for 10 min. The supernatant (1 mL) was mixed with 1 mL distilled water and 0.2 mL 0.1% ferric chloride $FeCl_3$, and the absorbance was measured at 670 nm after setting in the dark at room temperature for 10 min. The control is included by replacing the sample volume by equal volume of solvent.

Free radical scavenging activity

Antioxidant activity was measured using 2, 2-Diphenyl- 1-picrylhydrazyl (DPPH) radical-scavenging assay as described by Brand-Williams et *al.* [13] where a fixed volume of each hydrolysate (0.1mL) was mixed with 3.9 mL of 6.10^{-5} M of methanol DPPH solution, *the mixture* was allowed to stand in the *dark* at room temperature for 30 min, the absorbance was read at 515 nm. The control was prepared by replacing the sample with distilled water.

The free radical scavenging activity (inhibition %) was calculated from following equation:

$$\text{Scavenging activity (inhibition \%)} = \text{abs}_{\text{control}} - \text{abs}_{\text{sample}} / \text{abs}_{\text{control}}) * 100$$

Blank sample and reference standard consist of methanol and BSA respectively.

Total antioxidant capacity (TAC)

Determination of antioxidant capacity, through the formation of phosphomolybdenum complex is performed as decribed by Prieto et *al*. [14]. 0.1 mL of sample (20 mg) solution is combined with 1 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The aqueous solutions are incubated in a boiling water bath at 95 °C for 90 min. After cooling, the absorbance is measured at 695 nm against blank.

β-carotene/linoleic acid bleaching test

The method is assed according to Wanasundra et *al*. [15]. Briefly 1 mL of chloroformic solution of β carotene was pipetted into round bottom flask and 20µl of linoleic acid, 200 mg of tween 40 emulsifer were added. After removing chloroform by rotary evaporator at 40°C, 50 mL of oxygenated water was added to flask and shaken, aliquots of 5 mL of the emulsion were added to 0.5 mL of each sample, and immediately placed in water bath at 50°C. The absorbance was read against blank at different time intervals for 120 min at 470 nm.

Antiviral activity of hydrolysates against Cox B6

The antiviral activity was assayed against Coxsackie virus, the multiplications of infection (MOI) were 0.1 and 1 MOI, using monocellular layers 50000 for Vero and 30000 for A549 cells per well in 96 well microplates for 72h. 100µl from each hydrolysate concentrations (0.035-120 µg/mL) were added to 40µl of virus suspension and 100µl of DMEM containing 7 and 10% FCS for A549 and Vero cells respectively, after incubation 1h at 37°C. Cellular suspension was added, and plates were incubated at 37°C in 5% CO_2. At the end of the period of 72h, viable cells were revealed by neutral red and estimated by reading the absorbance at 540 nm using plate reader [16]. The percentage of antiviral activity is calculated as followed:

$$\mathbf{AA= OD_S- OD_V OD_C- OD_V}$$

OD_S is the optical density of the cells infected by virus and protected by the hydrolysates.
ODv is the optical density of the cells infected by virus.
ODc is the optical density of the cells protected by the hydrolysates and not infected by virus

Statistical analysis

All the tests were performed in triplicate and the graph was plotted with mean value.

RESULTS

Casein was isolated from acidified defatted milk. For determining the total level of protein in a solution, the *Lowry* protein assay was performed and the camel milk casein concentration was found 30.81g.L^{-1}.

Polyacrylamide gel electrophoresis (SDS- PAGE)

Molecular weight of casein and its pepsin hydrolysates were compared to molecular standard (Fig. 1).

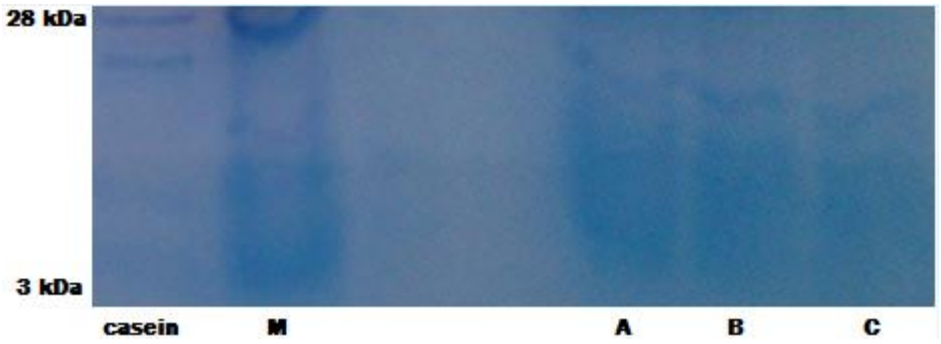

Fig. 1. *SDS page of camel milk casein and its pepsin hydrolistes at different hydrolysis time* ***M****: molecular weight marker, peptic hydrolysate at 60min* ***(A)*** *peptic hydrolysate at 90min* ***(B),*** *peptic hydrolysate at 120min* ***(C).***

Reducing power assay

Antioxidant peptides produced by pepsin hydrolysis of camel milk casein have reducing power. The absorbance increases up to 90 min (Fig. 2).

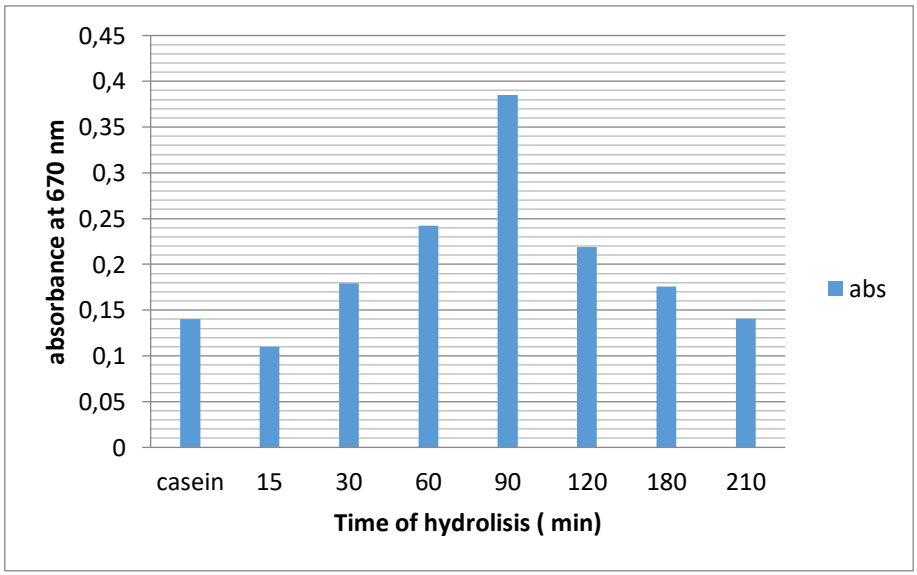

Fig. 2. *Dependence of reducing power of hydrolysis products of casein with pepsin of various time of hydrolysis.*

DPPH free radical scavenging activity

Scavenging activity of camel casein hydrolysates are determined using DPPH radicals (Fig. 3). The antioxidant activity goes increasing to 120 min and after that decreases. Increasing the degree of hydroyisis of casein leads to decrease in activating effect of peptides.

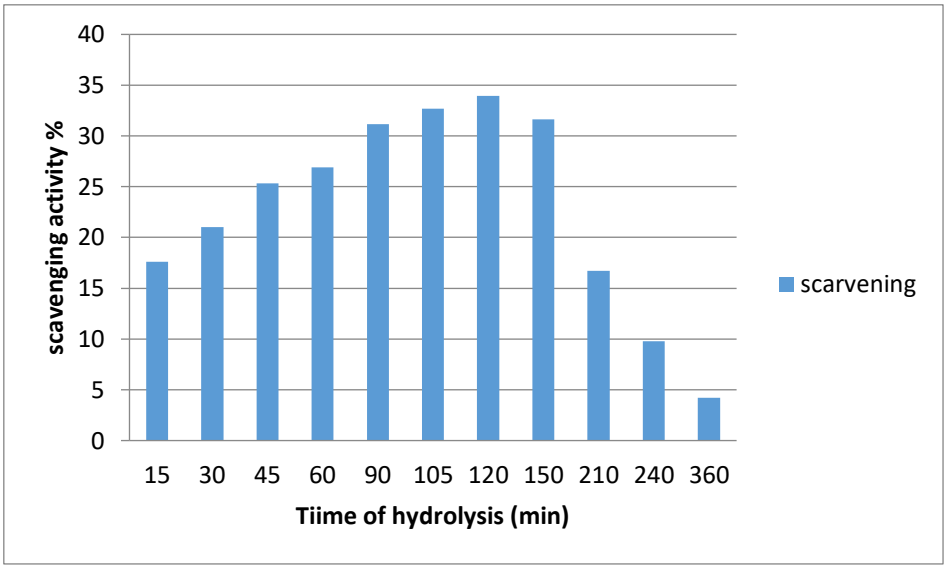

Fig. 3. *Free radical scavenging activity DPPH activity of casein hydrolysates by pepsin at different time of hydrolysis*

Total antioxidant capacity (TAC)

To evaluate the total antioxidant capacity of peptide generated from hydrolysis, the reduction of phosphate Mo (VI) to phosphate Mo (V) by the sample and subsequent formation of a bluish green colored phosphate/Mo (V) complex at acid pH. The absorbance goes increasing till 180 min of hydrolysis (Fig. 4).

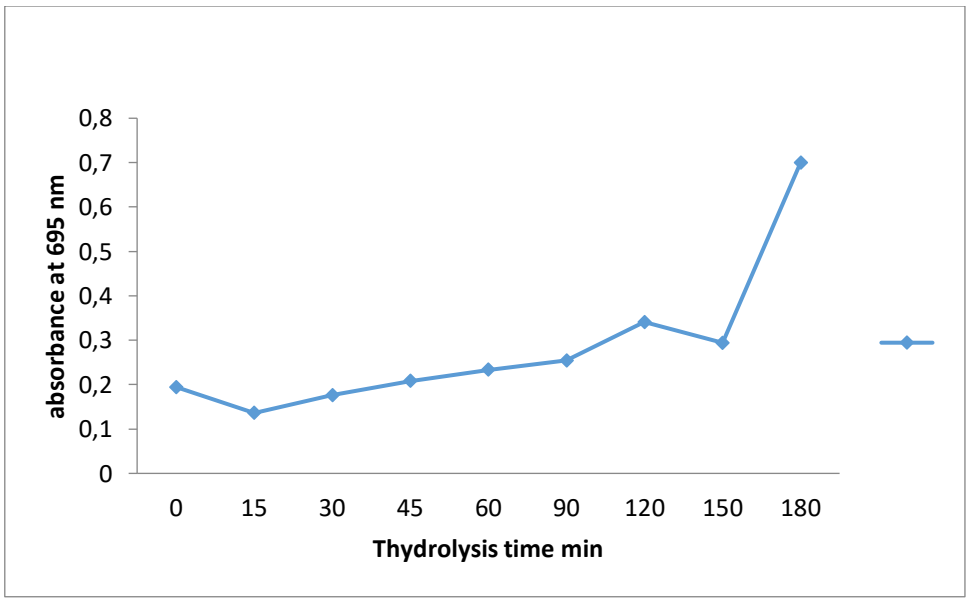

Fig. 4. *Dependence of the absorbance on the time of hydrolysis in phosphomolibdenum essay*

β-carotene/ linoleic acid bleaching test

The comparative absorbances of the hydrolysates at different time intervals till 120 min as described in the procedure of this assay indicates that hydrolysates of 120 and 90 shows higher values (Fig. 5).

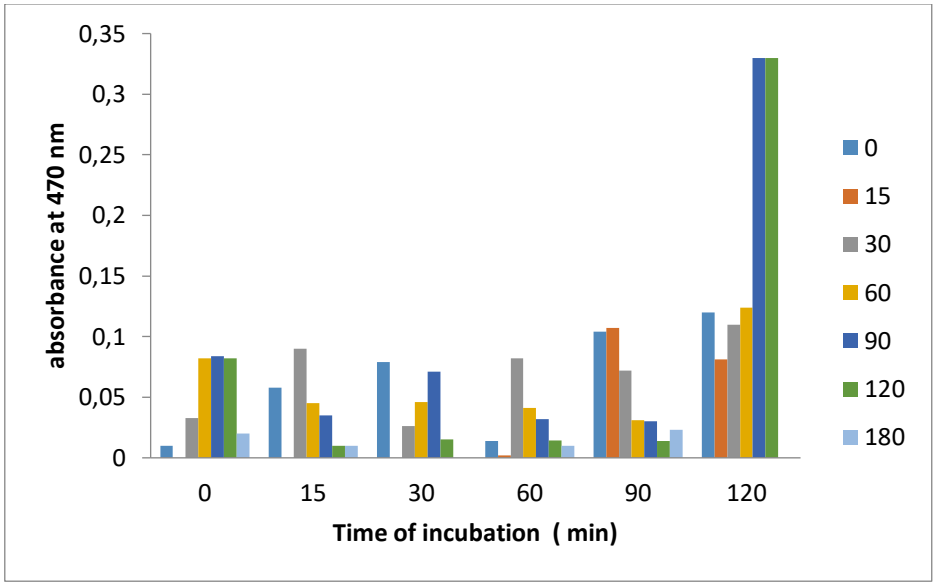

Fig. 5. *Bleach ability of β carotene in linoleic acid system at different time intervals*

Antiviral activity against Cox B6

The antiviral activity of camel milk casein hydrolysates against Cox B6 results are shown in Fig 6-11.

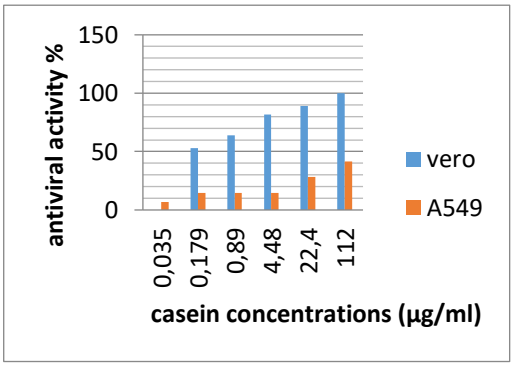

Fig. 6. *Antiviral activity of casein, incubated with Cox B6 1 MOI*

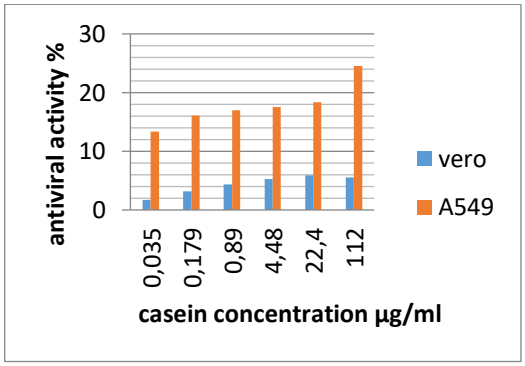

Fig. 7. *Antiviral activity of casein, incubated with Cox B6 0.1 MOI*

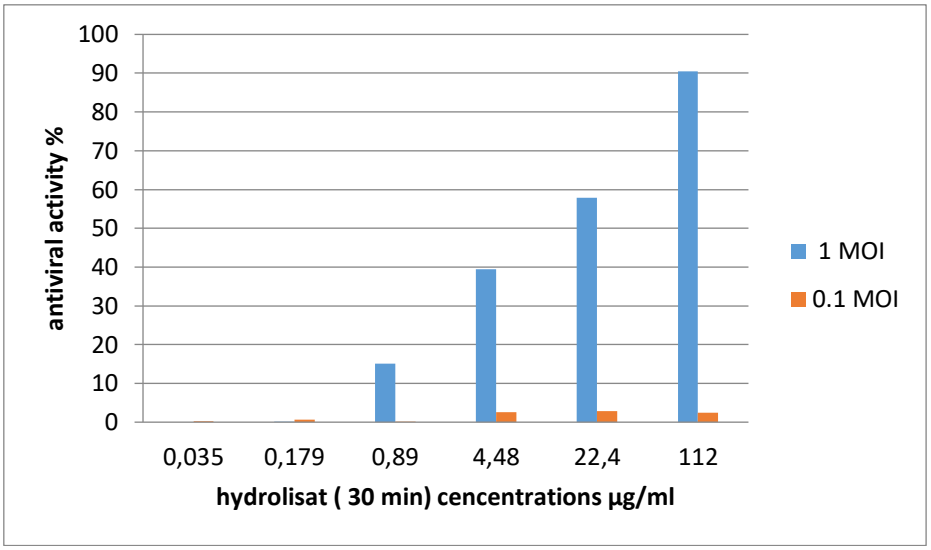

Fig. 8. *Antiviral activity of casein hydrolisat (30 min) ,incubated with Cox B6 (1MOI) effect on A549 cell*

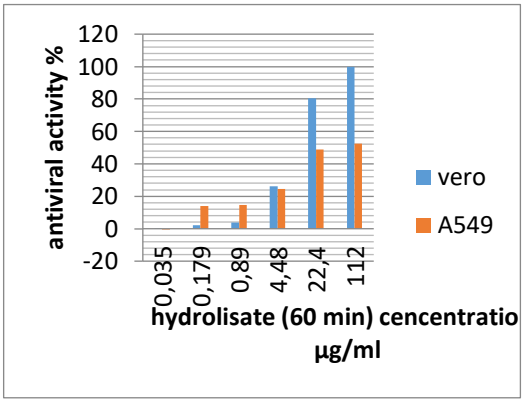

Fig. 9. *Antiviral activity of hydrolisate at 60 min incubated with Cox B6 1MOI*

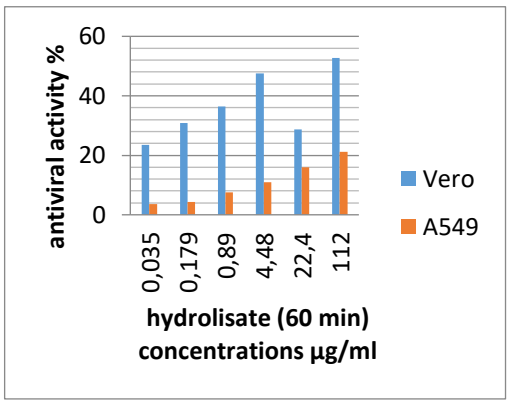

Fig. 10. *Antiviral activity of hydrolysate at 60 min incubated with Cox B6 0.1 MOI*

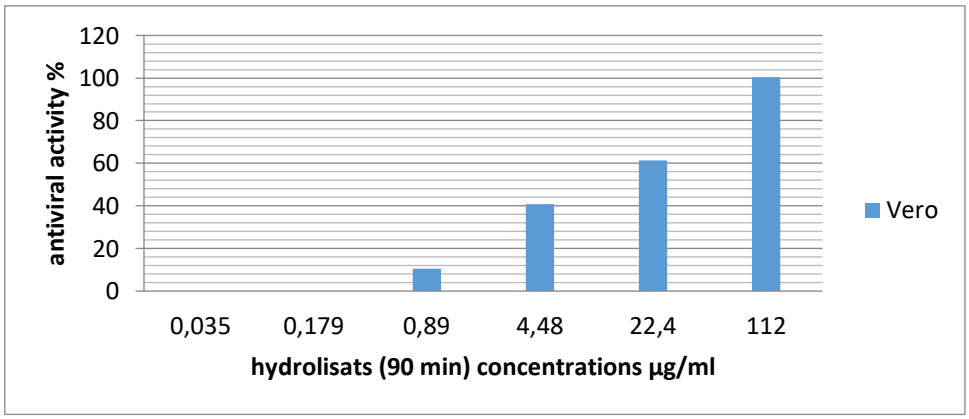

Fig. 11. *Antiviral activity ofhydrolisate at 90 min incubated with Cox B6 (1 MOI)*

DISCUSSION

Fat was removed from camel milk and, after precipitation by acidification the casein obtained was isolated, the bands in SDS-page give an estimation of molecular weight of casein approximately 28 kDa, it was reported that camel mil casein has a weight of α s1-CN and β - CN 33 and 29.5 kDa respectively **[**17], another study mentioned 31, 25, and 27 kDa for α s1 –, α s2 – and β- casein respectively [18], after hydrolysis bands of smaller molecular weight are visible.

The time hydrolysis goes up to 180 minutes, the biological activity of peptides depends on the protein substrate, enzyme specificity, and hydrolysis conditions.

Reducing power assay

This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in the reducing power. In this method, antioxidant compound forms a colored complex which is measured at 670 nm.

The presence of deductants in peptic camel milk hydrolysate up to 90 min, causes the reduction of the $Fe^{3+/}$ferricyanide complex to the ferrous form. Therefore, the Fe^{2+}can be monitored by measuring the formation of blue at 695 nm. For the pepsin treatment hydrolysate produced by 90 min hydrolysis, the greatest reducing power is expedited. The peptide cleavages increase the availability of protons and electrons resulting from enzymatic hydrolysis. [19].

DPPH free radical scavenging activity

The DPPH activity of camel milk casein hydrolysates has been used to evaluate its antioxidant activity, relatively high DPPH scavenging activity was observed with different enzymes at a longer incubation time where the α chymotripsyne at 2h of hydrolysis produced hydrolysates which had significantly higher antioxidant activity up to 6 h [20]. The antioxidant activity increased significantly with the progress in hydrolysis time up to 120 min where the higher activity is about 33.97 %, then decreases. Relationship between hydrolysis time which act like electron donor and DPPH activity could be established where scavenging activity of pepsin hydrolysates may be a result of its higher enzymatic activity.

β-carotene/ linoleic acid bleaching test

The of β carotene /linoleic acid bleaching test is usually used to detect antioxidants like phenolics, in this study, casein hydrolisates showed antioxidant property, up to 120 min of incubation, the hydrolysates have an inhibitory activity against linoleic acid auto oxidation,

Its antioxidant activity is due to increased accessibility to hydrophobic targets [21]. In fact, hydrophobic amino acids in the camel milk casein hydrolysates, which have higher affinity to the lipid phase than hydrophilic amino acids, can scavenge lipid-derived radicals [22].

Total antioxidant capacity (TAC)

This assay is a quantitative method to investigate the reduction reaction rate among antioxidant. It involves in thermally generating auto oxidation during prolonged

incubation period at high temperature, electron transfer occurs in this assay which depends upon the structure of the antioxidant [14].

Antiviral activity against Cox B6

Camel milk casein and its hydrolysate of 60 min have an antiviral activity in both Vero and A549 cell line, while its hydrolysate of 30 min shows an antiviral activity in A549 cell line only. Hydrolysate of 90 min also inhibits the infection of Vero cell line by Cox B6 but not A549 (data not shown).

The antiviral activity of camel milk hydrolysates has been investigated against the ability of Cox B6 to infect Vero and A549 cells. The time of incubation with virus, the concentration of the hydrolysates and the viral load (MOI) determine the amplitude of antiviral activity.

The concentration of 112 µg/mL of casein has the highest antiviral activity, with 41.33 and 100% efficiency against Cox B6 at concentration of 1MOI but decreases to 24.5 and 5.55% at 0.1 MOI, in A549 and Vero cells, respectively.

Hydrolysates with antiviral activity are 30, 60 and 90 min of hydrolysis, after that no activity was observed (data not shown). The camel milk casein hydrolysate of 30 min exhibits an antiviral activity, with 90.52% efficiency against Cox B6 at a concentration of 1 MOI and only 2% at 0.1 MOI in A549 cell line only, while the efficiency of hydrolysate of 60 min (112 µg/mL) is 52.78 and 100% at 1 MOI, and 21.13 and 52.6 % at 0.1 MOI in A549 and Vero cells respectively, also the 90 min hydrolysate shows a high antiviral acticity at a concentration of 112µg/mL with Vero cell only attending 100%.at 1MOI. Sitohy et *al*., [23] conclude that antiviral activity of esterified whey protein could be due to the perturbation of viral RNA- protein interaction, hence inhibition of the translation of viral proteins. With Cox B6 the level of reduction of viral genomic RNA reached 14%. Also, it was reported that pepsin cleaves peptide bonds involving hydrophobic and aromatic amino acids residues. Camel β-Casein has 46 possible potential cleavages [24]. Antiviral inhibitory effects are explained by the entry of hydrophobic inhibitory molecules in the hydrophobic binding cavities of the viral surface [25, 26], which may explain the blockage of Cox B6 cell entry after 1h of incubation by the hydrolysates and induce their antiviral activity in our study.

CONCLUSION

On the basis of the results obtained in the present study it can be concluded that camel milk casein hydrolysates presenting antioxidant activity were produced through hydrolysis with pepsin, had different structures based on SDS–PAGE, especially hydrolysates of 60, 90 min.

Antioxidant properties measured using DPPH free radical scavenging, reducing power, total antioxidant capacity and β carotene/ linoleic bleaching test were improved after hydrolysis of camel milk casein. These compounds are able to protect Vero and A549 from harmful viral infection (Cox B6). The results suggest the presence of biologically active peptide fractions, which may be worth for further investigation. Further work using other hydrolysis enzymes and animal models will be needed to conclude valuable information.

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